Journal of Biomolecular Structure & Dynamics, ISSN 0739-1102
Volume 24, Issue Number 5, (2007)

The Novel Approach to the Protein Design: Active Truncated Forms of Human 1-CYS Peroxiredoxin

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Abstract

The object of the present study is the verification of a new approach to the design of the active truncated forms of enzymes. The method is based on a new way of investigating the protein sequences – the ANalysis of Informational Structure (ANIS). The analysis of informational structure allows to determine the hierarchically organized structures (IDIC-trees) formed by the sites with the Increased Degree of Informational Coordination between residues. The proposed approach involves the consequent removal of the fragments corresponding to the individual IDIC-trees from the wild-type enzyme sequences. The described procedure was applied to the design of the active truncated form of human 1-CYS peroxiredoxin (PrxVI). Two variants of the PrxVI truncated sequences were proposed according to ANIS method. These truncated forms of the enzyme were expressed in *E. coli* and purified. The respective antioxidant activities were measured. It was shown that one of the truncated recombinant proteins retains more than 90% of the wild-type PrxVI enzymatic activity. According to the results of our study we can assume that ANIS method can be an effective tool for the design of the active truncated forms of the enzymes or the chimeric proteins which combine the enzymatic activities of their wild-type prototypes.

Introduction

The design of protein molecules with pre-determined enzymatic activity is one of the main problems in modern biotechnology. The first step in resolving that problem is an identification of functional fragments of the primary protein structure. The "one domain – one function" conception is now widely accepted, and the development of methods revealing functionally and structurally independent elements (domains) of proteins is of great importance. The most straightforward approach is to determine the spatial protein structures by X-ray diffraction and NMR spectroscopy. These methods, however, are expensive, time-consuming (X-ray) or subject to certain technical limitations (NMR). Therefore, various methods of analyzing the primary protein structure are usually applied to determine the functionally active domains. Here we report the application of a new method based on ANalysis of Informational Structure (ANIS) of the amino acid sequence to the identification of the functionally and structurally independent fragments (domains) of hPrxVI.

ANIS method is based on the two peculiar characteristics of the informational entropy function discovered earlier (1) by analyzing non-homologous sets of protein sequences, specifically:

1. The dependence of informational entropy on the inter residue distance in protein sequences is an S-shaped function. This indicates the existence of sequence fragments (up to dozens of residues in

Abbreviation: IS, Informational structure.

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length) possessing an Increased Degree of Informational Coordination (IDIC), or IDIC-sites.

2. The informational entropy values are small and constant at the distances over the six amino acid residues indicating a high degree of inter-residue correlation at the short distances. Therefore, primary structures of proteins may be regarded as series of short fragments: Information Units (IU).

Based on representation of amino acid sequences in terms of IU, a method has been developed for the identification of sequence IDIC-sites. The method was described previously (2). Various length IDIC-sites form the hierarchically organized informational structure of a protein sequence. For a number of proteins structural domains were shown to be corresponding to higher-order elements (IDIC-associations) of their Informational Structures (IS) (2).

In the present study, functionally active truncated forms of human peroxiredoxin VI have been designed by calculating and analyzing the IS of this protein in order to identify the functionally and IDIC-associations. According to such an informational analysis the variants of truncated hPrxVI forms have been created by the removal of the sequence fragments corresponding to adjacent IDIC-associations.

Peroxiredoxins (Prx) are a family of thiol peroxidases, which was found in bacteria, archea, unicellular organisms, higher plants, and animals. The Prx enzymes, which are localized to cytosol, mitochondria, plastids (chloroplasts), and peroxisomes, protect the cell by reducing H_2O_2 and oxygen radicals (3, 4). Peroxiredoxins are widely represented in cells, and the Prx family belongs to the top five of the most abundant cellular proteins (5).

Mammalian peroxiredoxins are divided into six isoforms, Prx (I-VI) (5) depending on the number of conserved Cys residues and the mechanism by which the sulphenic acid derivative of the essential Cys is catalytically converted to thiol. In spite of their different molecular sizes and catalytic mechanisms, the active sites of Prx I-VI are similar. A distinctive structural feature of Prx is the conserved part of the N-terminal domain (residues 44-54), where the Cys47 residue is surrounded by Pro and Thr (3, 6).

Peroxiredoxin VI, or 1-Cys peroxiredoxin, is the unique member of the Prx family that contains the only conserved Cys residue (Cys47) (7). This protein is highly abundant in tissues contacting atmosphere being a component of a primary defense system (7-10). We proposed possible therapeutic applications of Prx VI (hPrxVI), as a constituent part of pulmonary drugs (9, 11). In order to avoid possible side effects particularly immune reaction to exogenous protein, and to simplify the isolation and purification, full-sized enzymes can be advantageously replaced by their fragments retaining some of the selected biochemical activity specifically antioxidant properties.

Results and Discussion

Informational-structural Analysis of the hPrxVI Sequence

The IS of a protein sequence can be most efficiently represented by a diagram demonstrating areas with an increased degree of informational coordination (IDIC) between the residues. The IDIC-diagram of a protein displays its informational structure and helps to visualize the hierarchy of the structurally independent elements of the polypeptide chain. The IDIC-diagram of hPrxVI is shown on Figure 1. The IS of hPrxVI described below utilizes the terminology and notation that were proposed earlier (2). There are four IDIC-associations, {^{2}A16, ^{2}F37, ^{1}F43,

^{8}N65}, {^{7}A111}, {^{9}N156}, and {^{7}T208}, in the informational structure of hPrxVI. Remarkably three of those, {^{7}A111}, {^{9}N156}, and {^{7}T208}, are single IDIC-trees of a high rank. The first-rank IDIC-branches are centered at Val20, Gly30, Leu36, Phe43, Gly52, Ala55, Leu57, Glu60, Ala70, Ser75, Leu80, Ile86, Glu96, Ile102, Asp105, Ile112, Leu117, Ala120, Asp123, Ala131, Val133, Lys144, Thr152, Ile160, Val164, Leu167, Leu169, Glu172, Gly184, Ala198, Thr208, Leu211, Ser223, and Thr221. The corresponding branches of IDIC-trees, as determined by decomposition function half-width value of 2, are shown on Figure 1. Among the IS peculiarities there are six segments of the polypeptide chain with exceptionally high density of first-rank IDIC-branches, (Gly52, Ala55, Leu57, Glu60), (Ile102, Asp105), (Leu117, Ala120, Asp123), (Ala131, Val133), (Ile160, Val164, Leu167, Leu169, Glu172), and (Thr208, Leu211, Ser213). These segments with closely spaced centers of the first-rank IDIC-branches indicate the critical importance of the local spatial organization of such regions for the protein function. Such elements usually serve as the sites of intermolecular interaction (Nekrasov A., unpublished data). Each of the IDIC-associations {^{2}A16, ^{3}F37, ^{1}F43, ^{8}N65} and {^{7}T208} contains one segment of the described type, whereas each of the IDIC-associations {^{7}A111} and {^{9}N156} contains two sites of exceptionally dense first-rank IDIC-branches. It should be noted that the (Gly52, Ala55, Leu57, Glu60) branch is situated in the close vicinity of the Cys47 residue in the active site of hPrxVI.

Since the crystal structure of hPrxVI has been solved earlier (12), it can be visually compared to the IS of this protein calculated by the ANIS method (2). The elements of hPrxVI spatial structure corresponding to different IDIC-associations are shown in color on Figure 2. It can be seen that the hPrxVI molecule is "dumbbell"-shaped. The C-terminal domain (Lys182 – Pro224) contains IDIC-association {^{7}T208}. The N-terminal domain is structurally more complex and includes two IDIC-associations, {^{2}A16, ^{3}F37, ^{1}F43, ^{8}N65} and {^{7}A111}. IDIC-association {^{9}N156} spans over several elements of the spatial structure including two β-strands (Val133 – Gly138, Val175 – Pro178) of two different β-sheets and α-helix (Asp158 – Glu172) bridging the N- and C-terminal domains. Thus, IDIC-associations are well correlated with the actual elements of hPrxVI spatial structure.

The Structure and Design of hPrxVIΔ124 and hPrxVIΔ178

The data described above is useful for designing possible variants of the hPrxVI forms truncated from the C-terminus (Fig. 1). Since there is Cys47 located in the active site, truncated hPrxVI analogs must retain the sequence fragment corresponding to IDIC-association {^{2}A16, ^{3}F37, ^{1}F43, ^{8}N65} for the activity of enzyme to be preserved. To clarify the role of other individual IDIC-associations in preserving the hPrxVI activity, we deemed it necessary to obtain differently truncated analogs, the one including two IDIC-associations, {^{2}A16, ^{3}F37, ^{1}F43, ^{8}N65} and {^{7}A111}, and the other including the {^{9}N156} association, additionaly. The corresponding truncated proteins, hPrxVIΔ124 and hPrxVIΔ178, which include three (Gly52, Ala55, Leu57, Glu60; Ile102, Asp105; Leu117, Ala120, Asp123) and five (Gly52, Ala55, Leu57, Glu60; Ile102, Asp105; Leu117, Ala120, Asp123; Ala131, Val133; Ile160, Val164, Leu167, Leu169, Glu172) segments, respectively, are characterized by exceptionally high density of first-rank IDIC-branches. Note that the full-sized hPrxVI contains six such segments.

Assuming that truncated hPrxVI forms should preserve all the amino acid residues, which correspond to the first-rank IDIC-branches of their respective IDIC-associations, that protein may be truncated at residues located between any adjacent IDIC-associations. One of the deletion mutants (hPrxVIΔ124), which contains two IDIC-associations, was obtained by substituting Gln124-coding triplet by Stop codon, while the other analog (hPrxVIΔ178) – by replacing the triplet coding for Pro178. The two truncated hPrxVI forms were expressed as it was described previously for the human recombinant 1-Cys peroxiredoxin (13).

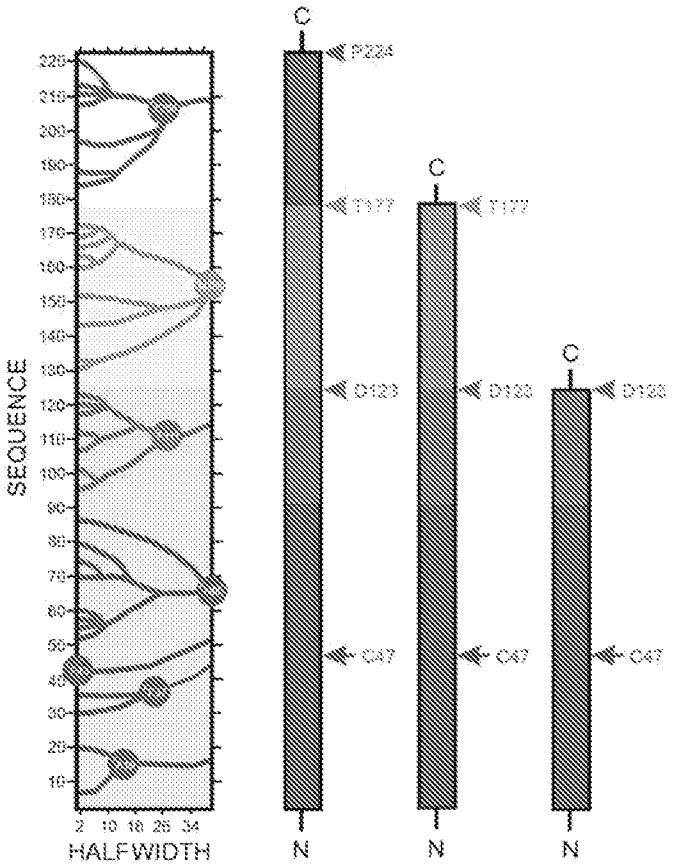


Figure 1: The IDIC-diagram of hPrxVI informational structure and linear schemes of the native and truncated protein sequences. The IDIC-associations and the corresponding primary structure segments are color-coded as follows: {^{1}A16, ^{2}F37, ^{3}F43, ^{4}N65}(red), {^{7}A111}(blue), {^{8}N156}(green), and {^{9}T208}(violet). The axes of the IDIC-diagram are amino acid residue numbers and the half-value of decomposition function. The stepwise truncation of hPrxVI sequence is indicated by the progressive darkening (from white to dark gray) of the background. The essential C47 residue of the active site and the C-terminal residues (P224, T177, and D123) of the full-sized hPrxVI and of the truncated proteins (hPrxVIΔ178 a hPrxVIΔ124) are indicated on the linear schemes of the respective sequences.

Fragments of hPrxVI cDNA coding 123 or 177 residues of the N-terminus were cloned into pET23-a(+) plasmid (Novagen) using *Nde* I – *Eco*R I restriction sites (see ***Materials and Methods***). pET23-a(+) (hPrxVIΔ124) and pET23-a(+) (hPrxVIΔ178) plasmids containing open reading frames under the control of the T7 phage promotor and including the respective hPrxVI cDNA fragments were used for further transformation of *E.coli* BL-21(DE-3) cells (Stratagene).

Recombinant proteins with the expected molecular weight were found in the water-soluble protein fraction of cell lysates obtained by ultrasonication. Electrophoretic separation of water-soluble proteins from cell lysate before and after IPTG induction is shown in Figure 3. Comparison of lanes 1, 3, and 4 indicates that the IPTG expression induction was followed by intensive accumulation of ~19 kDa and ~13 kDa proteins. The yields of both proteins were ~60 mg/1 L of the cell culture.

Since there is a 92% sequence homology between the rat and human PrxVI, the truncated proteins were identified by Western blot analysis using polyclonal rabbit antibodies against a 28-kD secretory protein from rat (14). Immunochemical data indicate that the ~19-kDa (lane 3) and ~13-kDa (lane 4) proteins, which concentration in *E. coli* cells sharply increases after the IPTG induction, are the truncated hPrxVI forms, hPrxVIΔ178 and hPrxVIΔ124, respectively (Fig. 3). Their calculated molecular weights are 19.6916 and 13.6915 kDa, and correspond well to the experimental values obtained by SDS-PAGE.

The recombinant proteins were purified by the method described earlier for PrxVI from rat (15). See ***Materials and Methods*** for the details. We used the chromatographic purification on a Sephacryl S-300 column to examine the behavior of hPrxVIΔ124 and hPrxVIΔ178. The hPrxVIΔ124 protein migrates together with the total column volume and shows a molecular mass of ~14 kDa, which is in agreement with those obtained from SDS gel and primary structure. The elution volume for hPrxVIΔ178 was about 60% of the total column volume, which corresponds to the elution time of polypeptides with the molecular weight of ~35 kDa [the results were obtained by a linear regression analysis of standard protein markers (not shown)]. This value does not agree with that obtained by SDS-PAGE but corresponds to the value predicted for the dimer form of hPrxVIΔ178.

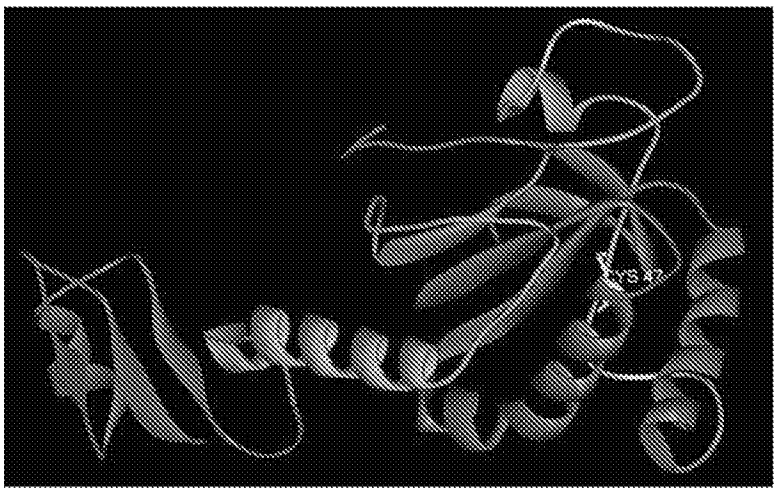


Figure 2: The spatial structure of the native hPrxVI. The structural elements corresponding to IDIC-associations {^{1}A16, ^{2}F37, ^{3}F43, ^{4}N65}, {^{7}A111}, {^{8}N156}, and {^{9}T208} are color-coded by red, blue, green, and violet, respectively. The ball-and-stick model representation is used for the essential C47 residue of the active site.

The native PrxVI from rat has been shown to prevent the inactivation of *E. coli* Glutamine Synthetase in a model oxidative system (14). The same test has been applied to hPrxVIΔ124 and hPrxVIΔ178 to determine their protective properties. Isoforms were compared to the full-sized recombinant hPrxVI, which is functionally identical to the native protein (13). In case of hPrxVIΔ178, a considerable protective effect was observed at protein concentration of 60 μg/ml (>60% of Glutamine Synthetase activity) whereas at 120 μg/ml of hPrxVIΔ178 100% of Glutamine Synthetase activity was preserved (Fig. 4a). The activity of hPrxVIΔ178 can be compared with the activity of the full-sized hPrxVI. The experiments with hPrxVIΔ124 in the range of hPrxVIΔ124 concentrations between 20 and 120 μg/ml showed the absence of protective effect, since Glutamine Synthetase was inactivated completely.

The measurements of the peroxidase activity of the recombinant hPrxVI fragments showed similar results, *i.e.*, the expressed hPrxVIΔ178 possessed the peroxidase activity (Fig. 4b). However, no peroxidase activity could be detected in case of hPrxVIΔ124. The lack of functional activity of hPrxVIΔ178 can be explained by the difference in the IS between hPrxVIΔ178 and hPrxVIΔ124. hPrxVIΔ124 does not contain IDIC-association {°N156} spanning between two fragments of the amino acid sequence with exceptionally high density of first-rank IDIC-branches. Thus, the hPrxVIΔ178 deletion mutant lacks only one site, (Thr204, Leu207, and Ser209), with exceptionally high density of first-rank IDIC-branches, whereas hPrxVIΔ124 lacks three such sites, (Ala131, Val133), (Ile160, Val164, Leu167, Leu169, Glu172), and (Thr208, Leu211, Ser213). Note that the largest high-density site in all of the hPrxVI sequence, (Ile156, V160, Leu163, Leu165, Glu168), is also absent in hPrxVIΔ124. Taking into account the results of gel filtration, we believe that this deletion mutant is practically incapable of dimerization, and {°N156} association works as the site of the intermolecular interaction. Consequently, dimerization of hPrxVI may be regarded as a functionally critical property of this protein.

The loss of the dimerization capability in the truncated form hPrxVIΔ124 correlates well with the lowered protective and peroxidase activity. Besides, one residue (Arg132) belonging to the IDIC-association {°N156} in the spatial structure of hPrxVI, is located only 3.76 E away from the sulphur atom of the essential Cys47 residue. The absence of Arg132 may further decrease the enzymatic activity of hPrxVIΔ124 (12).

Therefore, we believe that the truncated hPrxVI form could be used in antioxidant drugs instead of full-sized enzyme.

A new method of protein sequence analysis was applied in the present study to identify structurally and functionally independent fragments (structural domains) of the primary structure. The ANIS method can be an effective tool for the design of novel protein molecules (truncated and/or chimerical) that preserve or combine the functional properties of corresponding native proteins. The method may be especially useful for protein engineering when the spatial structure of the native prototype is unknown.

Materials and Methods

Data Collection and Informational Structure Detection

The informational structure analysis of hPrxVI is based on the interpretation of protein sequences in terms of IU as it has been described previously (2). The IDIC-diagram of hPrxVI has been constructed and analyzed using the following parameters and statistics (2).

In general, the informational structure analysis and construction of IDIC-diagrams follow a stepwise procedure:

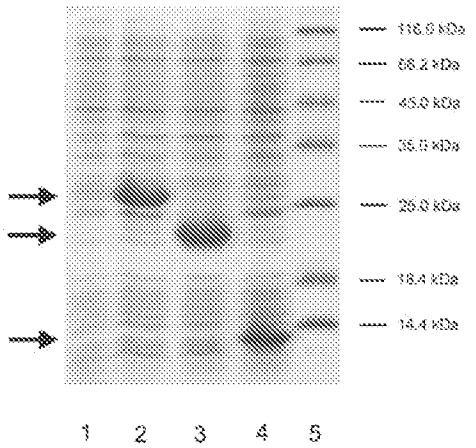
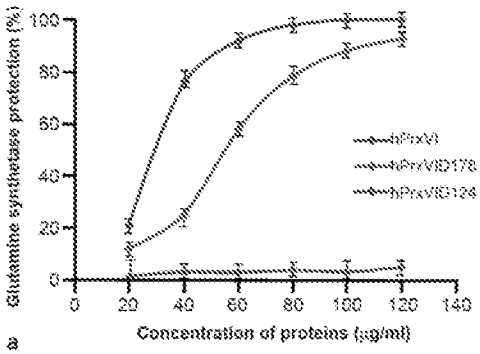


Figure 3: The analysis of cDNA expression of hPrxVI and its truncated fragments. 15% SDS-PAGE of the total cell protein from *E. coli* BL-21(DE-3) cells containing the initial pET23-a(+) plasmid (*1*); pET23-a(+) hPrxVI (*2*), pET23-a(+)hPrxVIΔ178 (*3*), and pET23-a(+)hPrxVIΔ124 (*4*) 6 h after IPTG induction; and marker proteins (*5*) with the molecular weights as shown. Arrows on the left indicate the positions of the recombinant proteins.



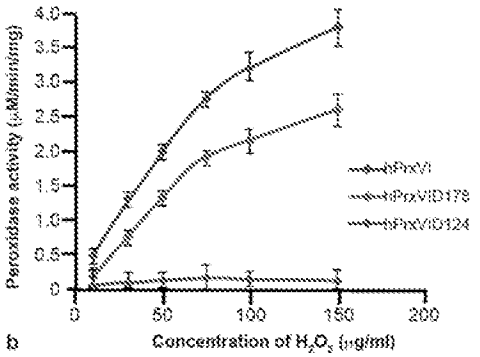


Figure 4: The protective (**a**) and peroxidase (**b**) activities of hPrxVIΔ178 and hPrxVIΔ124. (**a**) the glutamine synthetase (5μg) was incubated with 3 mM DTT, 3 μM $FeCl_3$, along with hPrxVIΔ124, hPrxVIΔ178, or hPrxVI as a control. The time of inactivation reaction was 10 min. (**b**) 10 μg hPrxVIΔ178, hPrxVIΔ124, or hPrxVI as a control was incubated in buffer with varying H_2O_2 concentrations and then remaining H_2O_2 was determined.

I. Re-coding of the amino acid sequence in terms of "information units";
II. Calculation the "information content" profile of the protein sequence of the interest based on the statistics of the "appearance density" of information units in model sets of non-homological protein sequences;
III. Detection the polypeptide chain segments of increased interrelation between the information units and constructing the IDIC-diagram of the informational structure.

Cloning and Expression of Truncated hPrxVI cDNA

A fragment of hPrxVI cDNA with non-altered open reading frame in the expression vector has been previously cloned (13). This vector (pET23-a(+)hPrxVI) was used as a PCR template. The obtained hPrxVI cDNA fragments were used for coding for two N-terminal fragments of hPrxVI, comprising 123 and 177 amino acid residues. In both cases, 5'-GCG AAA TTA ATA CGA CTC ACT ATA GGG-3' was used as a forward primer (corresponding to the promoter pET23-a(+)hPrxVI). The backward primers were 5'-CCA TCC TTC GAA TTC AAC **TTA** GGT GGC-3' for hPrxVIΔ178 (the *Eco*R I restriction site is underlined, the Stop codon is in boldface font) and 5'-GTC AGA GGA ATT CCC TTT **TAA** TCC-3' for hPrxVIΔ124 (the *Eco*R I restriction site is underlined, the Stop codon is in boldface font). hPrxVI cDNA fragments were cloned into pET23-a(+) plasmid (Novagen) using *Nde* I-*Eco*R I sites.

E.coli BL-21(DE-3) strain (Stratagene) containing T7 RNA polymerase gene under control of inducible *lac*-promotor (16) was used as a host strain for the expression of hPrxVI cDNA fragments. For the production of recombinant proteins, the cells were grown at 37 °C until the optical absorbance (A_{600}) of the cultivation medium reached 0.6 at 600 nm. Then the protein expression was triggered by adding *lac*-promotor inducer (IPTG) to the final concentration of 0.4 mM, and the media was further incubated for 5-6 h.

Isolation and Purification of hPrxVI Fragments

To produce the recombinant proteins at preparative scale, the cultivation media volume was increased to 250 ml. The cells were centrifuged at 4,000 g for 20 min at +4 °C. Cell pellet was resuspended in ice-cooled PBS buffer (140 μM NaCl, 2,7 μM KCl, 10 μM Na_2HPO_4, 1.8 μM KH_2PO_4, pH 7.3) by adding 50 μl of buffer for 1 ml of the cell culture. The resulting suspension was frozen, thawed, and sonicated on an ice bath (5 × 1 min, with 1 min pauses) at 200-300 W using a UP 50 H Ultrasonic Processor (Dr. Hielscher). The suspension was centrifuged at 5,000 g for 10 min at +4 °C, the resulting supernatant was additionally centrifuged at 35,000 g for 40 min at +4 °C. All further procedures were performed at this temperature. The supernatant was dyalized against 12 μM Tris-HCl buffer, pH 7.8, containing 1 μM $MgCl_2$ and 1 μM DTT, and the lysate was chromatographed on DEAE-Sepharose in a NaCl gradient as described earlier for the native rat peroxiredoxin. The deletion mutants were finally purified by gel filtration on a Sephacryl S-300 column (1 × 93 cm) as described earlier for the intact native protein (15). For hPrxVIΔ178, $V_e \approx 44$ ml; for hPrxVIΔ124, $V_e \approx 70$ ml. The purified protein fragments gave immunological cross-reaction with polyclonal rabbit antibodies against PrxVI from rat.

Enzymatic Activity of Peroxiredoxins In Vitro

Glutamine Synthetase was purified from *E.coli* strain DH5α as described previously (17) and inactivated by the model DTT/Fe^{3+}/O_2 oxidative system generating H_2O_2. The inactivation reaction was performed in a reaction mixture (60 μl) containing 5 μg of Glutamine Synthetase, 50 mM Hepes-NaOH buffer (pH 7.4), 3 mM DTT, and 3 μM $FeCl_3$ at various Prx concentrations within 10 min at 37 °C. The residual activity of *E. coli* Glutamine Synthetase was measured as described earlier (18). The protective effect of the peroxiredoxins against inactivation of *E.coli* Glu-

tamine Synthetase was defined as the ratio between the residual Synthetase activity after its inactivation in the presence of different peroxiredoxin concentrations and the activity of the native enzyme.

Peroxidase activity of the proteins was determined by direct measurement of the decrease in H_2O_2 level. The reaction was started by addition of 0.5 mM H_2O_2 in 50 mM HEPES buffer (pH 7.5) at 25 °C. At an appropriate reaction time, 0.1 ml of the reaction mixture was added to 0.05 ml of 0.6 M HCl to stop the reaction and when the 0.1 ml of 10 mM $Fe(NH_4)_2(SO_4)_2$ and 0.05 ml of 2.5 M KSCN was added to develop the chromogenic complex. The concentration of H_2O_2 was evaluated by spectrophotometric method at the wavelength $\lambda = 480$ nm.

Acknowledgments

We thank Dr. Vladimir Ovcharenko for helpful discussions and critical reading of the manuscript.

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Date Received: May 26, 2006

Communicated by the Editor Valery Ivanov